Translational Oncology
www.transonc.com

Volume 2 Number 3 September 2009 pp. 157–163 157

Altered microRNA Expression Patterns in Hepatoblastoma Patients[1]

Armando Magrelli*,[2], Gianluca Azzalin†,[2], Marco Salvatore*,[2], Mara Viganotti*, Fabrizio Tosto*, Teresa Colombo†, Rita Devito‡, Alessandra Di Masi§, Antonio Antoccia§, Stefano Lorenzetti¶, Francesca Maranghi¶, Alberto Mantovani¶, Caterina Tanzarella§, Giuseppe Macino† and Domenica Taruscio*

*National Centre for Rare Diseases, Istituto Superiore di Sanità, Rome, Italy; †Department of Cellular Biotechnology and Hematology, La Sapienza University, Rome, Italy; ‡Bambino Gesù Hospital, IRCCS, Rome, Italy; §Department of Biology, University Roma Tre, Rome, Italy; ¶Department of Food Safety and Veterinary Public Health, Unit of Veterinary and Food Toxicology, Istituto Superiore di Sanità, Rome, Italy

Abstract

Liver cancers in children are rare representing only 1.1% of malignancies, with an annual incidence rate of 1.5 cases per million. Hepatoblastoma and hepatocellular carcinomas are the most common malignancies of the liver occurring in young people aged 15 years or younger. Molecular basis of both tumors are still unclear, and common markers (i.e., *CTNNB1*, *APC*, *IGF-2*) are not always useful in the characterization of sporadic forms; in this respect, microRNA recently associated with carcinogenesis could play a pivotal role in their onset. *CTNNB1* and *APC* were analyzed by sequencing, and *IGF-2* promoter methylation status was assessed by methylation-specific polymerase chain reaction. MicroRNA expression was assayed by microarray and quantitative reverse transcription–polymerase chain reaction in hepatoblastoma samples. Although few genomic alterations were detected in ours samples, an altered expression of some microRNA in hepatoblastoma was observed. Unsupervised clustering shows that microRNA profile can distinguish tumor from nontumor tissues. Further analyses of microRNA contents in hepatoblastoma compared with hepatocellular carcinoma highlighted four upregulated microRNA (miR-214, miR-199a, miR-150 [$P < .01$], and miR-125a [$P < .05$]) and one downregulated microRNA (miR-148a [$P < .01$]). In conclusion, although our samples were poorly informative from a genetic point of view, they showed a peculiar microRNA expression pattern compared with nontumor tissues and hepatocellular carcinoma. MicroRNA could represent valid markers for the classification of pediatric liver tumors.

Translational Oncology (2009) 2, 157–163

Introduction

Hepatoblastoma (HB) and hepatocellular carcinoma (HCC) are two different subtypes of primary tumors arising from liver parenchymal cells [1]. Hepatoblastoma is a rare liver cancer with an incidence in western countries of 1.5 cases per million of individuals younger than 15 years. Despite this, it is the most common hepatic malignancy during childhood [1–3]. By contrast, HCC is a common solid cancer in adults: it is a complex and heterogeneous malignancy with several genomic alterations. Aberrant activation of several signaling cascades,

Address all correspondence to: Dr. Domenica Taruscio, National Centre for Rare Diseases, Istituto Superiore di Sanità, Viale Regina Elena, 299 – 00161 Rome, Italy.
E-mail: domenica.taruscio@iss.it

[1]This work was performed within the frame of the project "Tackling rare diseases yet lacking diagnosis and/or prognosis: a pilot project integrating data collection and experimental studies" supported by a NIH-ISS 2007–2009 grant.

[2]These authors have equally contributed to the studies presented in this manuscript.

Received 18 February 2009; Revised 16 March 2009; Accepted 16 March 2009

DOI 10.1593/tlo.09124

such as, phosphoinositol 3-kinase/mammalian target of rapamycin, Wnt, Hedgehog, insulin-like growth factor 2 (IGF-2), and apoptotic signaling have been extensively described in both HB and HCC [1,2].

Most cases of HB are sporadic, but children with familial adenomatous polyposis and Beckwith-Wiedemann syndrome have a higher risk for HB [1]. Although specific chromosome aberrations have not been linked with prognosis or casual factors, genetic disorders have been described in chromosomes 1q, 2q, 7q, 8, 17q, and 20 [4–6].

Activation of wingless (Wnt) signaling pathway through mutations in *β-catenin* (CTNNB1) may contribute to the development of HCC and HB [7–10]. *β-Catenin* is the central mediator of the well-known *Wnt/β-catenin* signaling pathway, which acts as a gene transcription (co)factor activating the T-cell factor/lymphoid enhancer factor [7,8].

In HB, mutations leading to constitutive activation of *β-catenin* have been identified in genes of the Wnt/β-catenin pathway: they occur with high frequency (48%-89%) in exon 3 of *CTNNB1* and are mainly characterized by deletions or point mutations [10]. Moreover, an increased level of gene expression of *IGF-2* compared with normal livers has been reported in a high proportion of human HB. In the fetal liver, promoters P2, P3, and P4 are active and expressed monoallelically; P3 is the most active promoter and P1 is inactive. However, in the adult liver, P1 becomes dominant and is biallelically expressed, and P2, P3 and P4 activities are decreased or lost [11]. In addition, P4 hypomethylation has been shown in several human cancers, including HCC, suggesting that it may be an early end common event in hepatocarcinogenesis [12]. Although the exact mechanism leading to these alterations is not entirely certain, it seems that loss of imprinting (LOI) and/or promoter demethylation are at least partly involved [13].

MicroRNA (miRs), an abundant class of noncoding small RNA ~21 nt, have been shown to play important roles in various cellular and pathogenic processes, including cellular development, immunologic response, and carcinogenesis [14–17]. Typically, they can modulate gene expression by regulating the messenger RNA at a post-transcriptional stage, aligning with sequences in the 3′-untranslated region [14,18–20]. MicroRNA can target oncogenes or tumor suppressor genes, contributing to the initiation and progression of many human cancers [21–23]. Several microRNA have been linked to initiation and progression of human cancers: miR-15a and miR-16 in chronic lymphocytic leukemia [24]; miR-145 in colon carcinoma, lung, breast, and prostate [25–29]; and miR-21 in glioblastoma [30]. Many well-known proteins involved in carcinogenesis are regulated by microRNA; for example, phosphatase and tensin homolog (PTEN) tumor suppressor is regulated by miR-21 and miR-214 [31,32], tumor suppressor protein $p27^{kip1}$ is regulated by miR-221 and miR-222 [33], *E2F1* transcription factor is regulated by the miR-17-92 cluster [34], and *BCL-2* antiapoptotic gene is regulated by miR-15a/miR-16 cluster [22].

Despite recent studies show that many microRNA are dysregulated in HCC [29,35–40] such as miR-145, miR-199a, miR-21, miR-34a, miR-221/222, and miR-224, no data are available on microRNA expression in HB patients yet.

In this study, we analyzed samples from nine HB patients, namely, three snap-frozen and six paraffin-embedded (FFPE) tissues. Gene expression profiling for microRNA on three snap-frozen matched samples was carried out to evaluate the global content of microRNA; results were validated by quantitative reverse transcription–polymerase chain reaction (qRT-PCR) in the same samples and in six additional matched (FFPE) tissues.

Materials and Methods

Tissue Samples and Clinical Data

The tissue samples analyzed in this study were obtained from patients undergoing a surgical procedure to remove a portion of the liver at the "Bambino Gesù" Hospital (Rome, Italy). Written informed consent for biologic studies was obtained from all patients analyzed, in accordance with the Declaration of Helsinki. Pathologic analysis by the institute providing the surgical specimens confirmed the histopathologic diagnosis of the samples taken for research.

We included in our study nine HB tumor samples (HBT) and their surrounding tissues not showing neoplastic features (from now called "normal tissue NT"). Their sex distribution was as follows: six males and three females (Table 1). All patients underwent SIOPEL chemotherapy treatment protocols. Six matched samples were FFPE (HBT1/NT1, HBT3/NT3, HBT5/NT5, HBT7/NT7, HBT9/NT9, and HBT11/NT11), whereas the three remaining samples were fresh-frozen (HBT13/NT13, HBT15/NT15, and HBT17/NT17). Tissues were collected from children aged between 8 and 39 months.

Nucleic Acid Extraction

DNA was extracted from samples using a standard proteinase K digestion and phenol/chloroform extraction; DNA was stored at +4°C in Tris-EDTA solution.

RNA was extracted using a standard Trizol RNA (Invitrogen, Milan, Italy) extraction method according to the manufacturer's instructions. The quality of nucleic acids was assessed by agarose gel electrophoresis, and concentrations were determined through Nanodrop ND-1000 (Thermo Scientific, Waltham, CA).

Mutational Analyses of DNA

DNA from samples was amplified by PCR with specific primers tailored for exon 3 of *CTNNB1* gene (*BCAT-1/BCAT-2*: *5′*-GATTTGATGGAGTTGGACATGG-*3′*; *5′*-TGTTCTTGAGTGAAGGACTGAG-*3′*) and exon 15 of *APC* gene (Fw: 5′-ACCTCCAACCAACAATCAGC-3′; Rev: 5′-CTTTGGATGACTGGGGAAAA-3′). After PCR amplification, products were purified using spin columns (QIAquick PCR purification kit; Qiagen, Inc., Milan Italy) and sequenced (CEQ 2000 XL DNA Analysis System; Beckman Coulter, Fullerton, CA).

Table 1. Clinical and Pathologic Characteristics of Patients with HB.

Case No.	Age (months)/Sex	Histologic Subtype*	*CTNNB1*†	*APC*‡	*IGF-2*§			
					P2A	P2B	P3	P4
HBT1	8/♀	Epithelial (F/E)	WT	WT	ND	ND	ND	ND
HBT3	39/♂	Epithelial (E)	WT	WT	ND	ND	ND	ND
HBT5	17/♂	Epithelial (F/E)	WT	WT	ND	ND	ND	ND
HBT7	34/♀	Epithelial (E)	WT	WT	ND	ND	ND	ND
HBT9	24/♀	Mixed	A43D	WT	ND	ND	ND	ND
HBT11	23/♂	Epithelial (F/E)	WT	WT	ND	ND	ND	ND
HBT13	11/♂	Epithelial (F)	T40S	WT	+/+	+/+	+/+	–/–
HBT15	6/♂	Epithelial (F/E)	WT	WT	+/+	+/+	+/+	–/–
HBT17	26/♂	Epithelial (F)	WT	WT	+/+	+/+	+/+	–/–

Tumor tissues examined in this study were collected at time of surgery or biopsy of primary treated tumors after chemotherapy.

*Predominant epithelial component is given in parentheses: *E* indicates embryonal differentiation only; *F*, fetal differentiation only; *F/E* indicates fetal and embryonal differentiation detectable.

†*β-Catenin* exon 3 characterization by sequencing: *WT* indicates wild type.

‡*APC* exon 15 characterization by sequencing: *WT* indicates wild type.

§*IGF-2* promoter methylation assay by MSP: *ND* indicates not determined; +, methylated; –, unmethylated.

For the detection of deletions, genomic DNA from HBT and NT tissues was amplified using the primer pairs BCAT-3/BCAT-4, BCAT-1/BCAT-6, flanking exons 2, 3, and 4 of the *CTNNB1* gene: *BCAT-3*, 5′-AAAATCCAGCGTGGACAATGG-3′; *BCAT-4*, 5′-TGTGGCAAGTTCTGCATCATC-3′; *BCAT-6*, 5′-GGAGGA-AGGTCTGAGGAGCAG-3′ [41].

Methylation-Specific PCR (MSP)

Methylation-specific PCR (MSP) was used to assess CpG site methylation within promoters (P) 2, 3, and 4 of the *IGF-2* gene: in particular, two MSP assays (P2A and P2B) were developed to evaluate methylation in P2. A lack of CpG sites within P1 limited its evaluation by MSP. Methylation-specific PCR was performed using the CpGenome DNA modification kit (Chemicon International, Milan, Italy). Briefly, 1 μg of genomic DNA was processed according to the manufacturer's instructions. Relative locations of the MSP assays and primers used in the assays are shown elsewhere [42].

μParaflo MicroRNA Microarray Assay

Microarray assay was performed on a custom array by a service provider (LC Sciences, Houston, TX). A 5-μg total RNA sample was extracted as previously described. Data were analyzed by first subtracting the background and then normalizing with a cyclic LOWESS filter (locally weighted regression). Interarray quantile normalization was performed using function "quantile.normalize" from R (www.r-project.org) package "affy" using default settings [43]. Finally, data were filtered to remove probes with constitutively low expression. Across patients, we required interquartile range for $\log_2$-transformed expression values across patients to be at least equal to 0.5 or higher and for expression to be equal to $\log_2(1000)$ or higher in at least two samples. Altogether, this procedure reduced the microRNA data set from 721 to 51 microRNA.

Cluster analysis was performed using the metric Euclidean distance to compute the distance matrix for HB patients based on the expression of 51 differentially expressed microRNA. To agglomerate the patients in the hierarchical cluster, we used the Ward method. Function *heatmap.2* from R package *gplots* was used for the graphical display of the dendrogram.

TaqMan Real-time RT-PCR

MicroRNA from FFPE tissues were extracted using mirVana miRNA Isolation kit (Ambion, Austin, TX). To evaluate mature microRNA in a specific manner, TaqMan MicroRNA assay kits were purchased from ABI (Foster City, CA) to detect miR-145 (catalog no. 4373133), miR-214 (catalog no. 4373085), miR-451 (catalog no. 4373209), miR-195 (catalog no. 4373105), miR-125a (catalog no. 4373149), miR-150 (catalog no. 4373127), miR-199a (catalog no. 4373272), miR-148a (catalog no. 4373130), and control RNA (RNU6B, cat. catalog no. 4373381). It is a two-step protocol requiring reverse transcription (catalog no. 4366536) with a microRNA-specific primer, followed by real-time PCR with TaqMan probes (catalog no. 4324018). The fold change of the microRNA gene in the tumor samples relative to the nontumor ones was calculated using the $2^{-\Delta\Delta CT}$ method. For details, refer to the ABI user's bulletin "Relative Quantitation of Gene

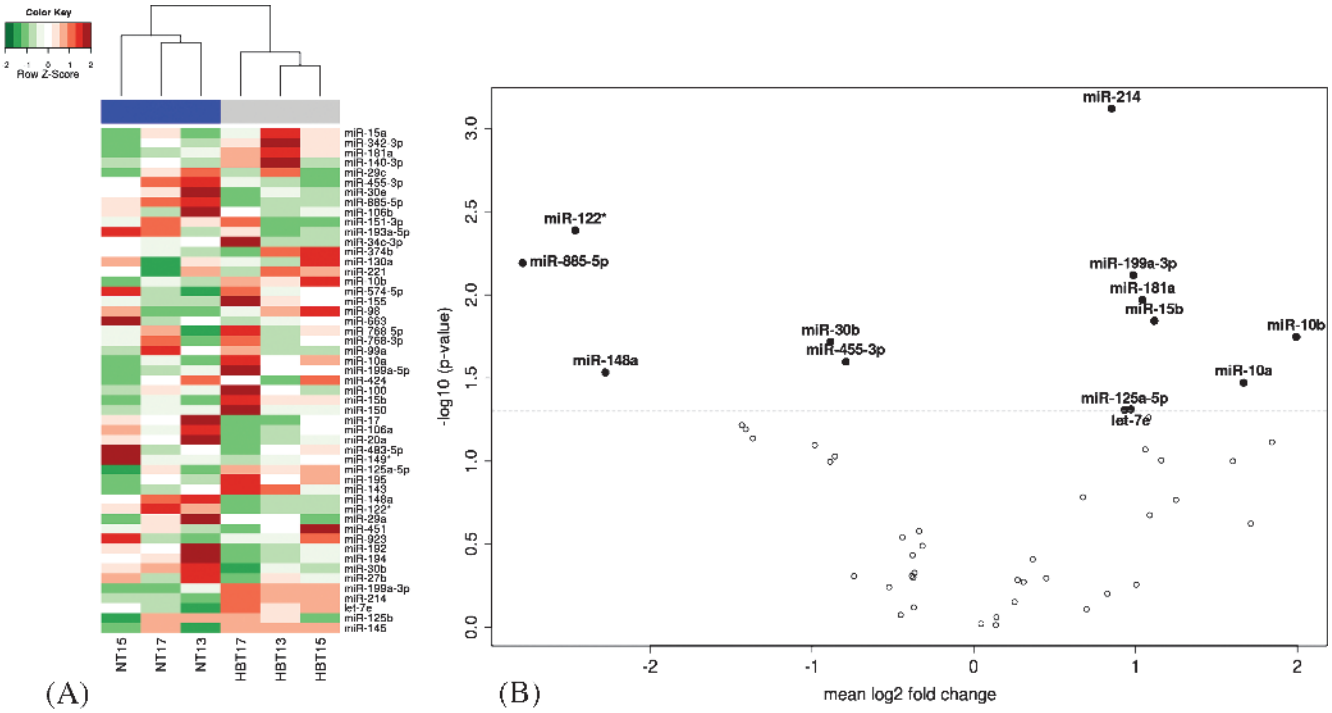

Figure 1. (A) Results of the unsupervised hierarchical clustering, in which NT tissues (blue) form a separate cluster from the HB tumors (gray). The heat map shows relative levels of microRNA expression in a green (low relative expression) to red (high relative expression) scale. (B) Volcano plot: a useful graphical that plots negative $\log_{10}$ *P* values (obtained from *t* test) on the *y*-axis *versus* mean $\log_2$ fold change on the *x*-axis, comparing altered microRNA expression in HB patients showing significant alteration of a small number of specific microRNA. Black dots indicate altered microRNA in HB *versus* NT tissues considered significant ($P < .05$).

Expression: ABI PRISM 7700 Sequence Detection System: User Bulletin 2: Rev B."

Pten transcripts were also evaluated using real-time PCR. One microgram of total RNA from each sample was reverse-transcribed using the TaqMan Reverse Transcription Reagents (Applied Biosystems). For the PCR analysis, *Gapdh* was used as an internal control: *Gapdh* primers (5′-GAA GGT GAA GGT CGG AGT C-3′ and 5′-GAA GAT GGT GAT GGG ATT TC-3′) were designed using the Primer Expression software (Applied Biosystems). The PCR primers for *Pten* (5′-CGGCAGCATCAAATGTTTCAG-3′ and 5′-AACTGGCAGGTAGAAGGCAACTC-3′) are described elsewhere [31]. Real-time PCR was performed using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). In a total volume of 20 μl of reaction mixture, 0.1 μg of complementary DNA templates were mixed with 10 μl of SYBR GREEN PCR Master Mix (Applied Biosystems) and each pair of primers at a final concentration of 100 nM. The thermal cycling conditions were set up sequentially as follows: denaturing at 95°C for 10 minutes and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Each sample was assayed in triplicates.

PTEN Western Blot

Frozen tissues were weighted and homogenized in an appropriate amount of lysing Buffer A (Sigma-Aldrich, St. Louis, MO), supplemented with 0.6 U/ml of benzonase (Merck KGaA, Germany) and protease inhibitor cocktails (Sigma-Aldrich) according to the manufacturers' instructions. After protein quantification by Bradford protein assay (Bio-Rad, Germany), 25 μg of whole protein extracts were loaded onto a 10% SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Immobilon, Millipore, Billerica, MA). The blots were probed with anti-PTEN monoclonal antibody (1:1000; Cell Signaling, Beverly, MA) and with 1 μg/ml anti–β-actin monoclonal antibody (Sigma-Aldrich) overnight at 4°C. After 1 hour of incubation with either an anti–rabbit or an anti–mouse secondary antibody (1:2000; GE Healthcare, United Kingdom), the enhanced chemiluminescence detection procedure was used to visualize antibody reaction (ECL Kit; GE Healthcare).

Results

β-Catenin, APC, *and* IGF-2 *Gene Characterization in HB Tissues*

Mutations in exon 3 of *β-catenin* and in *APC* gene are frequent in HB. We performed mutational analysis to identify possible mutations on both genes in our tissue samples.

Screening of HB samples showed a point mutation in HBT9 patient causing a A43D substitution and an additional point mutation in HBT13 causing a T40S substitution; no other point mutation and/or deletion were observed in *β-catenin* exon 3 and its flanking regions. We also performed mutational analysis on *APC* gene, and no mutations were detected in exon 15 in our HB samples (Table 1).

The *Igf2* gene transcription is driven by four promoters (P1-P4) that produce distinct transcripts which vary by tissue type and developmental stage. To describe *Igf2* promoter activity, we analyzed methylation status of selected CpG islands located in its promoters. The analysis was conducted in the three frozen matched samples (HBT13/NT13, HBT15/NT15, and HBT17/NT17). No differences were detected in P2, P3, and P4 promoters' methylation status pattern in our samples; in particular, both P2 and P3 promoters were found to be methylated in all HB samples. In contrast, P4 CpG island, within promoter 4, was hypomethylated (Table 1).

MicroRNA Expression Profile in Human HBs

MicroRNA-related studies on cancers are currently based on the different expression profile of microRNA in cancer *versus* normal tissue. Differential expression of the candidate microRNA is a possible approach to study the function of microRNA in cancer pathogenesis. To assess the putative role of microRNA in HB, we performed microarray microRNA analysis.

Three matched frozen samples were measured by microarrays in a cohort of nine matched samples of HB. Unsupervised clustering (Figure 1*A*), performed by using processed data from microRNA microarray analysis, shows tumor samples and adjacent nonmalignant tissues classified into two groups.

To identify the differentially expressed microRNA that cause clustering of tumor *versus* nontumor tissues, we calculated mean $\log_2$ ratio of the signal between HBT/NT. Levels of significance and magnitude of change in expression are represented in a volcano plot (Figure 1*B*); 13 differentially expressed microRNA, showing a good

Table 2. MicroRNA Showing Different Pattern of Expression in HB Compared with the HCC.

MicroRNA	Locus	Expression in HB Fold Change*	Expression in HCC	References
Upregulated				
hsa-miR-10	17q2132	1.67	Up	[38]
hsa-miR-10b	2q31.1	1.99	Up	[45]
hsa-miR-125a-5p	**19q133**	**0.93**	Down	[36]
hsa-miR-140-3p	16q22.1	1.25	ND	
hsa-miR-143	5q33.1	1.06	Down	[35]
hsa-miR-145	**5q331**	**1.16**	Down	[38]
hsa-miR-150	**19q133**	**1.71**	Down	[35]
hsa-miR-155	21q213	1.84	Up	[46]
hsa-miR-15	13q143	0.67	Up	[38]
hsa-miR-15b	3q26.1	1.12	ND	
hsa-miR-181	1q313/ 9q33	1.04	Down	[35]
hsa-miR-195	**17p131**	**0.93**	Down	[35,36,40]
hsa-miR-199a-3p	**1q243**	**0.99**	Down	[36]
hsa-miR-199a-5p	**1q243**	**1.60**	Down	[36]
hsa-miR-214	**1q243**	**0.85**	Down	[35,39,46]
hsa-miR-221	Xp113	1.00	Up	[35]
hsa-miR-342-3p	14q322	1.08	ND	
hsa-miR-574-5p	4p14	0.83	ND	
hsa-miR-923	17q12	0.70	ND	
hsa-miR-98	Xp1122	1.09	Up	[40]
hsa-Let-7*	19q1333	0.97	Up	[40]
Downregulated				
hsa-miR-106	**Xq262**	**−0.98**	Up	[47]
hsa-miR-122*	18q2131	−2.46	ND	
hsa-miR-148	**7p152**	**−2.28**	Up	[41]
hsa-miR-149*	2q373	−0.74	ND	
hsa-miR-17	13q313	−0.86	ND	
hsa-miR-192	11q13.1	−1.43	Up	[40]
hsa-miR-194	1q41/ 11q13.1	−1.41	NC	[40]
hsa-miR-20	13q313	−0.89	Up	[47]
hsa-miR-30b	8q2422	−0.88	ND	
hsa-miR-30	1p342	−1.37	ND	
hsa-miR-455-3p	9q32	−0.79	ND	
hsa-miR-885-5p	3p253	−2.79	ND	

MicroRNA differentially expressed in HBT *versus* NT samples. MicroRNA analyzed in the study and compared with data from literature (expression in HCC) are in bold face font.
*Values are calculated as mean[$\log_2$(HBT)] − mean[$\log_2$(NT)]: *Down* indicates downregulated in HCC *versus* NT tissues; *NC*, no change in the expression pattern; *ND*, no data are available in the literature; *Up*, upregulated in HCC *versus* NT tissues.

level of significance ($P < .05$), are indicated. Unsupervised clustering analysis using these 13 microRNA can discriminate tumor from nontumor tissues (data not shown), thus indicating that these microRNA could be a valid diagnostic for HB, although the small number of samples analyzed requires further future analyses.

Five MicroRNA Differentially Expressed in HB Showed a Different Pattern Compared with HCC

Recent evidences published on HCC point out that specific microRNA are involved in the onset and/or progression of the disease; our results were compared with different microRNA signatures published (Table 2). We validated, by qRT-PCR, the microRNA that showed a different pattern of expression compared with the HCC in all matched samples (Table 2). Analysis of selected microRNA in HB samples (Figure 2) showed that four of them were significantly upregulated in the tumor compared with the nonmalignant tissue (miR-214, miR-199a, miR-150 [$P < .01$], and miR-125a [$P < .05$]) and one was significantly downregulated (miR-148a [$P < .01$]). In contrast, miR-106a, miR-145, miR-195, and miR-451 were not significantly dysregulated (data not shown).

MicroRNA 214 and PTEN

The tumor suppressor *PTEN* is a negative regulator of the phosphoinositide 3-kinase signaling, and an alteration of this signaling

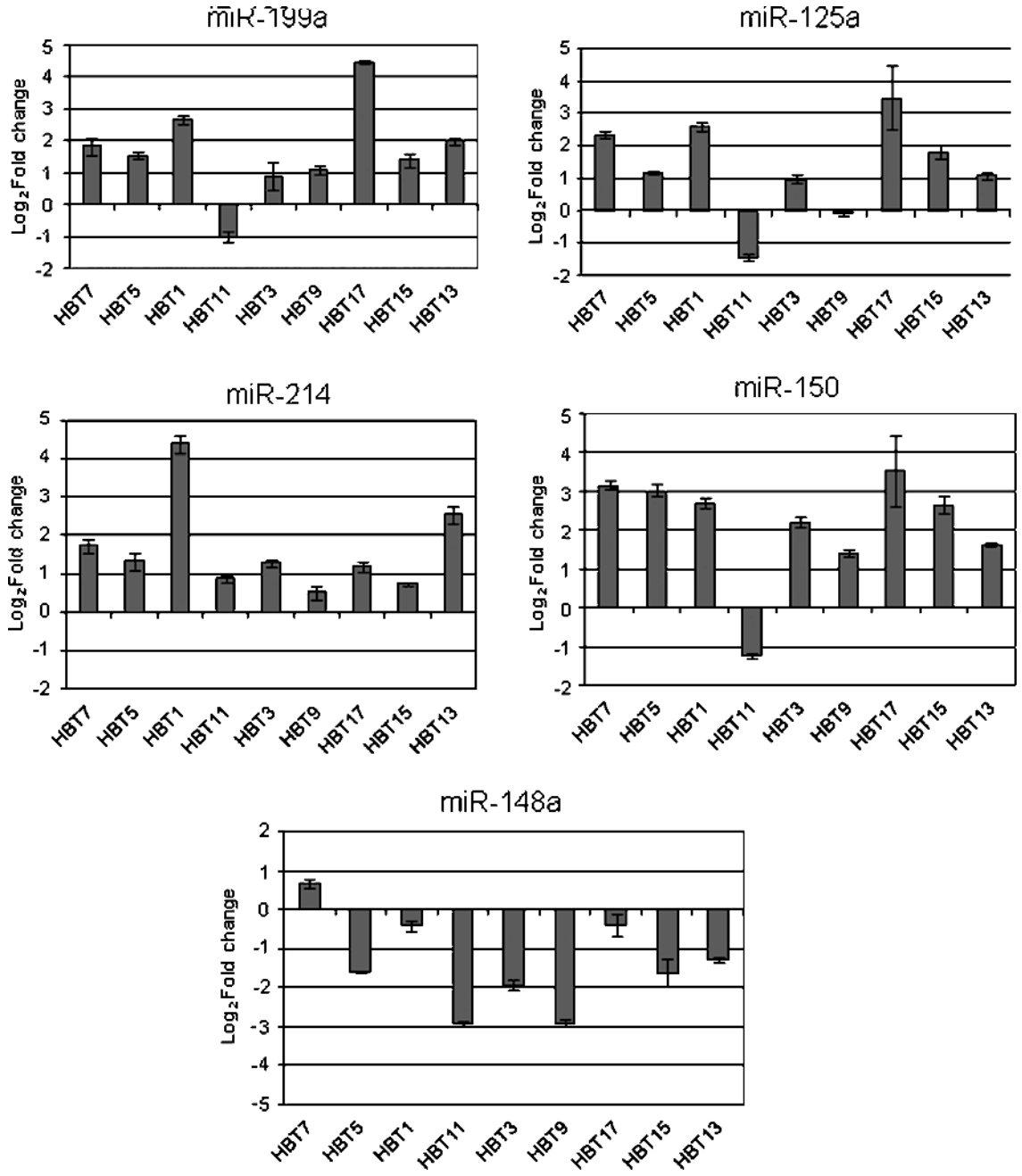

Figure 2. Validation of microRNA showing different pattern compared with HCC in nine HB patients and in nonneoplastic tissues. Each histogram plots U6 normalized $\log_2$ fold change (ratio of tumors to nontumors). Error bars, SD of three replicas.

cascade has been shown to give rise to different malignancies and cancer. Recent evidences show an active contribution of miR-214 and miR-21 in controlling *PTEN* protein level. To verify the involvement of miR-214 and miR-21 in HB samples, we measured miR-21 and miR-214 expression levels by qRT-PCR; although miR-21 was not significantly changed in all specimens, miR-214 showed an up-regulation in the tumor *versus* nontumor tissues (Figure 3*A*). Furthermore, to assess whether miR-214 exerts its function by transcript degradation or translational inhibition, *PTEN* protein levels were measured and found to be decreased in HBT13/NT13, HBT15/NT15, and HBT17/NT17 samples (Figure 3*B*), thus showing an inverse correlation with miR-214. In contrast, *Pten* messenger RNA was measured in all samples, and no significant alterations were detected (data not shown).

Discussion

Research approaches to pediatric rare tumors are strongly linked to the samples' availability, and although markers (i.e., *CTNNB1*, *APC*, *IGF2*) have been successfully used in the identification and classification of liver cancers, in our study, the analysis of these genes was not sufficient to characterize the differences between tumor and nontumor samples. In fact, only two mutations were identified in *CTNNB1* gene, and no LOI was observed in our samples [44].

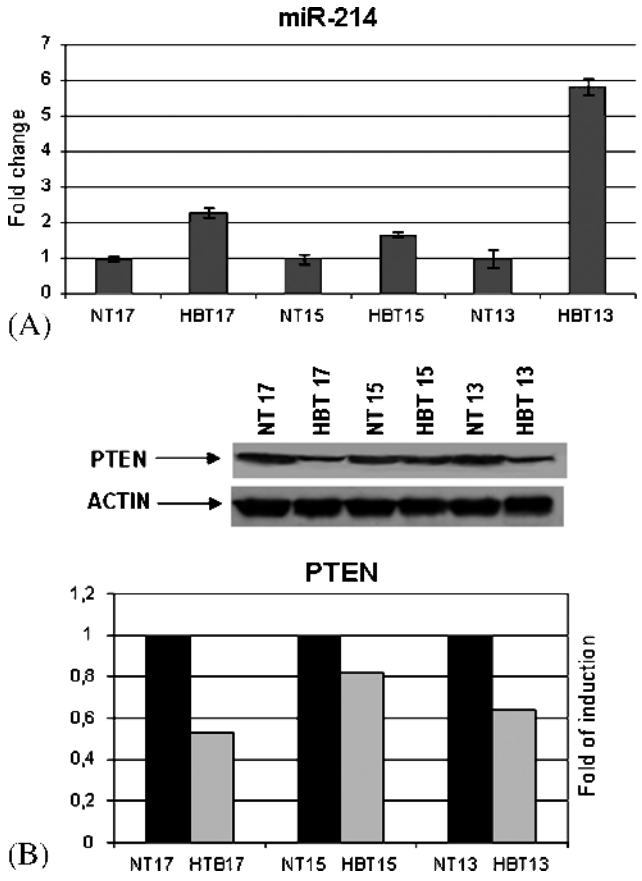

Figure 3. *PTEN* and miR-214 correlation of three patients from HB frozen samples and relative nontumor tissues. (A) Quantitative reverse transcription–polymerase chain reaction microRNA analysis. Bars display relative values of miR-214 expression in HB compared with nontumor tissues (taken to 1). Error bars, SD of three replicas. (B) Western blot analysis of variations in the expression of PTEN protein and relative densitometry analysis. *β-Actin* was used as loading control.

The low percentage of mutations in candidate genes and the absence of LOI observed in our study may be due to the limited number of samples, which often affects the analysis performed on rare tumors. To tackle this issue, we used microRNA as a new potential way to look for putative differences in rare cancers; actually, several reports indicate that microRNA expression profile is a feasible method for cancer classification [22].

In this study, global microRNA profiling identified differentially expressed microRNA that were commonly deregulated in HB patients.

We found that 33 of 51 filtered microRNA were differentially expressed: 21 of them resulted to be upregulated and 12 were downregulated. To the best of our knowledge, this is the first report about microRNA in HB patients. Moreover, we found that several microRNA showed a completely different behavior compared with HCC. In fact, although miR-214, miR-199a, miR-150, and miR-125a were upregulated in our matched samples, they were downregulated in HCC [38]. On the contrary, miR-148a was downregulated in HB and upregulated in HCC [40].

Interestingly, miR-214 has been demonstrated to induce cell survival in ovarian cancer through targeting the 3′-untranslated region of the *Pten*, thus leading to the down-regulation of its protein [32]. Other reports also indicate that *PTEN* protein levels are regulated by miR-21 in HCC [31]. *PTEN* is instrumental in regulating the balance between growth and death in several cell types because it regulates Akt signaling pathway and it is misregulated in several HB cases.

To assess the role of miR-214 or miR-21 in the regulation of *PTEN*, we tested their expression level in our samples; although no significant change in the miR-21 expression was observed, we found miR-214 to be significantly upregulated in all our samples.

Although no significant variation on the messenger level of *Pten* was detectable in tumors *versus* nontumor tissue samples, we observed a significant reduction of *PTEN* protein in the three matched frozen samples analyzed. We suggest that *PTEN* is negatively regulated by miR-214 at the protein level and that down-regulation of *PTEN* might correlate with elevated levels of miR-214.

Therefore, an active role of miR-214 in the activation of *PTEN*-mediated AKT pathway, previously described in ovarian cancer [32], could share common characteristics with other sporadic tumors; further investigations on independent cohort of samples are needed to shed light on the PTEN/AKT pathway and miR-214 in HB.

In conclusion, we pointed out for the first time that the expression pattern of 33 microRNA could represent a potential clue in HB pathogenesis, and in particular, 13 microRNA could be functional in discriminating tumor *versus* nontumor tissue. In addition, five microRNA (miR-214, miR-199a, miR-150, miR-125a, and miR-148a) were found to be helpful in discriminating HB from HCC, thus representing valid markers for the classification of pediatric liver tumors.

Future clarification of microRNA actions and functions will substantially improve our understanding of liver carcinogenesis. Development of drugs and molecules that specifically regulate hepatic microRNA with subsequent normalization of altered target expression may lead to novel treatments for liver cancers.

Acknowledgments

The authors thank Fiorentino Capozzoli for critical reading and comments.

References

[1] Roebuck DJ and Perilongo G (2006). Hepatoblastoma: an oncological review. *Pediatr Radiol* **36**, 183–186.

[2] McLaughlin CC, Baptiste MS, Schymura MJ, Nasca PC, and Zdeb MS (2006). Maternal and infant birth characteristics and hepatoblastoma. *Am J Epidemiol* **163**, 818–828.

[3] Reynolds P, Urayama KY, Von Behren J, and Feusner J (2004). Birth characteristics and HB risk in young children. *Cancer* **100**, 1070–1076.

[4] Suzuki M, Kato M, Yuyan C, Takita J, Sanada M, Nannya Y, Yamamoto G, Takahashi A, Ikeda H, Kuwano H, et al. (2008). Whole-genome profiling of chromosomal aberrations in hepatoblastoma using high-density single-nucleotide polymorphism genotyping microarrays. *Cancer Sci* **99**, 564–570.

[5] Nagata T, Nakamura M, Shichino H, Chin M, Sugito K, Ikeda T, Koshinaga T, Fukuzawa M, Inoue M, and Mugishima H (2005). Cytogenetic abnormalities in HB: report of two new cases and review of the literature suggesting imbalance of chromosomal regions on chromosomes 1, 4, and 12. *Cancer Genet Cytogenet* **156**, 8–13.

[6] Steenman M, Westerveld A, and Mannens M (2000). Genetics of Beckwith-Wiedemann syndrome–associated tumors: common genetic pathways. *Genes Chromosomes Cancer* **28**, 1–13.

[7] Hu M, Kurobe M, Jeong YJ, Fuerer C, Ghole S, Nusse R, and Sylvester KG (2007). Wnt/signalling in murine hepatic transit amplifying progenitor cells. *Gastroenterology* **133**, 1579–1591.

[8] Sangkhathat S, Kusafuka T, Miao J, Yoneda A, Nara K, Yamamoto S, Kaneda Y, and Fukuzawa M (2006). *In vitro* RNA interference against beta-catenin inhibits the proliferation of pediatric hepatic tumors. *Int J Oncol* **28**, 715–722.

[9] Mulholland DJ, Dedhar S, Coetzee GA, and Nelson CC (2005). Interaction of nuclear receptors with the Wnt/beta-catenin/Tcf signaling axis: Wnt you like to know? *Endocr Rev* **26**, 898–915.

[10] Koesters R and von Knebel Doeberitz M (2003). The Wnt signaling pathway in solid childhood tumors. *Cancer Lett* **198**, 123–138.

[11] Li X, Gray SG, Flam F, Pietsch T, and Ekström TJ (1998). Developmental-dependent DNA methylation of the IGF2 and H19 promoters is correlated to the promoter activities in human liver development. *Int J Dev Biol* **42**, 687–693.

[12] Tang SH, Yang DH, Huang W, Zhou HK, Lu XH, and Ye G (2006). Hypomethylated P4 promoter induces expression of the insulin-like growth factor-II gene in hepatocellular carcinoma in a Chinese population. *Clin Cancer Res* **15**, 4171–4177.

[13] Gray SG, Eriksson T, Ekstrom C, Holm S, von Schweinitz D, Kogner P, Sandstedt B, Pietsch T and Ekstöm1 TJ (2000). Altered expression of members of the IGF-axis in hepatoblastomas. *Br J Cancer* **82**, 1561–1567.

[14] Dalmay T (2008). MicroRNAs and cancer. *J Intern Med* **263**, 366–375.

[15] Abbott AL, Alvarez-Saavedra E, Miska EA, Lau NC, Bartel DP, Horvitz HR and Ambros V (2005). The let-7 MicroRNA family members mir-48, mir-84, and mir-241 function together to regulate developmental timing in *Caenorhabditis elegans*. *Dev Cell* **9**, 403–414.

[16] Lee CT, Risom T, and Strauss WM (2006). MicroRNAs in mammalian development. *Birth Defects Res C Embryo Today* **78**, 129–139.

[17] Chen CZ, Li L, Lodish HF, and Bartel DP (2004). MicroRNAs modulate hematopoietic lineage differentiation. *Science* **303**, 83–86.

[18] Lee RC and Ambros V (2001). An extensive class of small RNAs in *Caenorhabditis elegans*. *Science* **294**, 862–864.

[19] Lau NC, Lim LP, Weinstein EG, and Bartel DP (2001). An abundant class of tiny RNAs with probable regulatory roles in *Caenorhabditis elegans*. *Science* **294**, 858–862.

[20] Lagos-Quintana M, Rauhut R, Lendeckel W, and Tuschl T (2001). Identification of novel genes coding for small expressed RNAs. *Science* **294**, 853–858.

[21] Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, et al. (2005). MicroRNA expression profiles classify human cancers. *Nature* **435**, 834–838.

[22] Calin GA and Croce CM (2006). MicroRNA-cancer connection: the beginning of a new tale. *Cancer Res* **66**, 7390–7394.

[23] Esquela-Kerscher A and Slack FJ (2006). Oncomirs—microRNAs with a role in cancer. *Nat Rev Cancer* **6**, 259–269.

[24] Calin GA, Cimmino A, Fabbri M, Ferracin M, Wojcik SE, Shimizu M, Taccioli C, Zanesi N, Garzon R, Aqeilan RI, et al. (2008). MiR-15a and miR-16-1 cluster functions in human leukemia. *Proc Natl Acad Sci USA* **105**, 5166–5171.

[25] Yanaihara N, Caplen N, Bowman E, Seike M, Kumamoto K, Yi M, Stephens RM, Okamoto A, Yokota J, Tanaka T, et al. (2006). Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. *Cancer Cell* **9**, 189–198.

[26] Akao Y, Nakagawa Y, and Naoe T (2007). MicroRNA-143 and -145 in colon cancer. *DNA Cell Biol* **26**, 311–320.

[27] Akao Y, Nakagawa Y, and Naoe T (2006). MicroRNAs 143 and 145 are possible common onco-microRNAs in human cancers. *Oncol Rep* **16**, 845–850.

[28] Bandres E, Cubedo E, Agirre X, Malumbres R, Zárate R, Ramirez N, Abajo A, Navarro A, Moreno I, Monzó M, et al. (2006). Identification by real-time PCR of 13 mature microRNAs differentially expressed in colorectal cancer and non-tumoral tissues. *Mol Cancer* **5**, 29.

[29] Iorio MV, Ferracin M, Liu CG, Veronese A, Spizzo R, Sabbioni S, Magri E, Pedriali M, Fabbri M, Campiglio M, et al. (2005). MicroRNA gene expression deregulation in human breast cancer. *Cancer Res* **65**, 7065–7070.

[30] Chan JA, Krichevsky AM, and Kosik KS (2005). MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. *Cancer Res* **65**, 6029–6033.

[31] Meng F, Henson R, Wehbe-Janek H, Ghoshal K, Jacob ST, and Patel T (2007). MicroRNA-21 regulates expression of the *PTEN* tumor suppressor gene in human hepatocellular cancer. *Gastroenterology* **133**, 647–658.

[32] Yang H, Kong W, He L, Zhao JJ, O'Donnell JD, Wang J, Wenham RM, Coppola D, Kruk PA, Nicosia SV, et al. (2008). MicroRNA expression profiling in human ovarian cancer: miR-214 induces cell survival and cisplatin resistance by targeting *PTEN*. *Cancer Res* **68**, 425–433.

[33] Galardi S, Mercatelli N, Giorda E, Massalini S, Frajese GV, Ciafrè SA, and Farace MG (2007). miR-221 and miR-222 expression affects the proliferation potential of human prostate carcinoma cell lines by targeting $p27^{Kip1}$. *J Biol Chem* **282**, 23716–23724.

[34] Hayashita Y, Osada H, Tatematsu Y, Yamada H, Yanagisawa K, Tomida S, Yatabe Y, Kawahara K, Sekido Y and Takahashi T (2005). A polycistronic microRNA cluster, miR-17-92, is overexpressed in human lung cancers and enhances cell proliferation. *Cancer Res* **65**, 9628–9632.

[35] Gramantieri L, Ferracin M, Fornari F, Veronese A, Sabbioni S, Liu CG, Calin GA, Giovannini C, Ferrazzi E, Grazi GL, et al. (2007). Cyclin G1 is a target of miR-122a, a microRNA frequently down-regulated in human hepatocellular carcinoma. *Cancer Res* **67**, 6092–6099.

[36] Murakami Y, Yasuda T, Saigo K, Urashima T, Toyoda H, Okanoue T, and Shimotohno K (2006). Comprehensive analysis of microRNA expression patterns in hepatocellular carcinoma and non-tumorous tissues. *Oncogene* **25**, 2537–2545.

[37] Budhu A, Jia HL, Forgues M, Liu CG, Goldstein D, Lam A, Zanetti KA, Ye QH, Qin LX, Croce CM, et al. (2008). Identification of metastasis-related microRNAs in hepatocellular carcinoma. *Hepatology* **47**, 897–907.

[38] Varnholt H (2008). The role of microRNAs in primary liver cancer. *Ann Hepatol* **7** (2), 104–113.

[39] Jiang J, Gusev Y, Aderca I, Mettler TA, Nagorney DM, Brackett DJ, Roberts LR and Schmittgen TD (2008). Association of microRNA expression in hepatocellular carcinomas with hepatitis infection, cirrhosis, and patient survival. *Clin Cancer Res* **14**, 419–427.

[40] Huang YS, Dai Y, Yu XF, Bao SY, Yin YB, Tang M, and Hu CX (2008). Microarray analysis of microRNA expression in hepatocellular carcinoma and non-tumorous tissues without viral hepatitis. *J Gastroenterol Hepatol* **23**, 87–94.

[41] Honda S, Arai Y, Haruta M, Sasaki F, Ohira M, Yamaoka H, Horie H, Nakagawara A, Hiyama E, Todo S, et al. (2008). Loss of imprinting of IGF2 correlates with hypermethylation of the H19 differentially methylated region in hepatoblastoma. *Br J Cancer* **99**, 1891–1899.

[42] Koch A, Denkhaus D, Albrecht S, Leuschner I, von Schweinitz D, and Pietsch T (1999). Childhood hepatoblastomas frequently carry a mutated degradation targeting box of the *β-catenin* gene. *Cancer Res* **59**, 269–273.

[43] Beeghly AC, Katsaros D, Wiley AL, Rigault de la Longrais IA, Prescott AT, Chen H, Puopolo M, Rutherford TJ and Yu H (2007). IGF-II promoter methylation and ovarian cancer prognosis. *J Cancer Res Clin Oncol* **133**, 713–723.

[44] Gautier L, Cope L, Bolstad BM, and Irizarry RA (2004). affy—analysis of Affymetrix GeneChip data at the probe level. *Bioinformatics* **20**, 307–315.

[45] Ladeiro Y, Couchy G, Balabaud C, Bioulac-Sage P, Pelletier L, Rebouissou L, and Zucman-Rossi J (2008). MicroRNA profiling in hepatocellular tumors is associated to clinical features an oncogene tumor suppressor gene mutations. *Hepatology* **47** (6), 1955–1963.

[46] Wang Y, Lee AT, Ma JZ, Wang J, Ren J, Yang Y, Tantoso E, Li KB, Ooi LL, Tan P, et al. (2008). Profiling microRNA expression in hepatocellular carcinoma reveals microRNA-224 up-regulation and apoptosis inhibitor-5 as a microRNA-224–specific target. *J Biol Chem* **283** (19), 13205–13215.

[47] Kutay H, Bai S, Datta J, Motiwala T, Pogribny I, Frankel W, Jacob ST, and Ghoshal K (2006). Downregulation of miR-122 in the rodent and human hepatocellular carcinomas. *J Cell Biochem* **99**, 671–678.